Unknown Bacteria: Differentiating between Peptostreptococcus Anaerobius and Peptostreptococcus stomatis for the Clinic

An Honors Thesis (HONR 499)

by

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Abstract

To me science, as a concept, has always been the never-ending search for the "why" and "how" different elements around us do what they do. This concept appeals to highly detailed and inquisitive people that are not content to leave things as they are but want to search further for explanations. In a medical clinic these questions have paramount importance as their answers can have serious impact on the health and life of a patient seeking care. Medical practitioners need to understand what they are dealing with down to the microscopic level to give the best and most appropriate care possible to each patient. This, however, is a daunting task, especially with new research leading to new discoveries every day. Early in the new century a sample of microbes from the genus *Peptostreptococcus* was discovered to be two different species from the genus rather than the singular species the sample was originally believed to be. Consistent differentiation proved to be difficult between the two species as they were hard to culture, or grow in a lab, even though they are implicated in certain diseases. Our goal for this research was to design primers that could be used to easily identify, on a genetic level, an unknown microbe of *Peptostreptococcus* to allow for more precise treatment of a disease or infection. These primers would consist of complimentary genetic code for target gene sequences in the bacteria combined with SYBR green for fluorescence. If the primers anneal to the target sequences appropriately during polymerase chain reaction (PCR), then the fluorescence would be the indicator of the target gene's presence.

Acknowledgments

I would like to first recognize my partner in this project, Mark "Spencer" Goodman. It was he who approached me with the idea of undertaking a research project together and it is he who has been a stalwart companion throughout this process that has spanned over a year.

I would like to thank Dr. John McKillip for advising me through this project. It was in his classroom that the inspiration for this research became a reality and his guidance, encouragement, and willingness to exceed any and all expectations are a large part of how this project came to be and shaped how Spencer and I are as scientists today.

I would like to thank my parents, Jim and Gail, as well as my sister, Katie, for always being there for me and encouraging me to undertake new challenges.

Process Analysis Statement

This project definitely had humble beginnings. The idea for our research first came to be as an extension of a project assigned in Dr. McKillip's Microbiology (BIO 313) class that Spencer and I shared. The project dealt with the identification of a specific microbe from a real-world sample through the use of several determinative tests we had been discussing in lecture and lab. In Spencer's project, specifically, he had difficulty differentiating between two specific bacteria of the *Peptostreptococcus* genus. He approached me in March 2017 with the idea of creating an outside research project to find a new and effective way of differentiating between those two bacteria, *P. anaerobius* and *P. stomatis.* It was not until we approached Dr. McKillip about pursuing the research that he suggested that such a topic would be appropriate for a senior Honor's thesis.

Preliminary research on *P. anaerobius* and *P. stomatis* was slow to say the least. The two bacteria had only been distinguished as two separate species in the late 2000's so there was not much prior data on tests that would definitively differentiate the two. Since our project was first viewed as an extension of the classroom Microbiology project, we tried to model our research process based on the differentiating tests we had learned about among others we researched.

Thus, our first proposed method of research was to use cultures of the two bacteria and expose the two to iron shavings. We knew from our research that *P. anaerobius* produces isovaleric acid as an end product of metabolism while *P. stomatis* does not. Our thought process was that, since isovaleric acid is corrosive to metals, it will oxidize the iron shavings and, if there was observed degradation of the iron shavings placed into the culture, then that would be indicative of *P. anaerobius*. This, we believed, would be a cost-effective way for medical clinics to differentiate between the two bacteria as iron shavings would not be expensive to procure.

This is where the research project became real and exciting for the pair of us and could be considered our first major "breakthrough" in our project. For the first time we were moving away from the preliminary research stage, which had spanned several months, and moved into devising our own ways to solve the problem. It had been a long time coming, but we had devised what could have been our own small contribution to science and, even though this first method did not end up becoming the procedure we used for our project, it was still an important step in our path that would eventually take us to our results.

Eventually, however, we had to move on from our first method and think of a new way to differentiate the two bacteria. This came to be, ultimately, because we could not think of a way to quantify the amount of degradation in the iron shavings as a result of isovaleric acid production. *P. anaerobius* does not produce a large amount of isovaleric acid during its metabolism so, even if some iron is broken down in the culture, it would be difficult to measure, or even observe with the eye, any degradation. As a result, we decided to pursue differentiation based on the genetic material of the two bacteria.

In our research we learned that *P. anaerobius* had a higher content of genetic material than *P. stomatis,* so we figured that there would be some genes present in *P. anaerobius* alone. The new plan was to find three genes unique to *P. anaerobius* and design primers to match that material and indicate through PCR whether the specific sequences were present in a sample and, thus, distinguishing between the two bacteria. In the clinical setting, since the primers would already be designed from our research, it would be simple and cost-effective to obtain these primers and use them to identify the microbe sample in question. This ultimately turned out to be a better process than our first idea and became the method our research was based on.

Choosing proper DNA sequences unique to *P. anaerobius* took several hours searching through online libraries of gene sequences known to appear in the two bacteria. Our selections then had to be further narrowed down depending on their appropriateness and ability to design primers based on the sequence. Once the three sequences (*folP*, *galT*, and *galK*) were decided upon it was finally time to order the materials which included samples of the bacteria, components for our PCR "recipe," and media to house the samples. The functions of the three target genes include, for *folP*, the production of the protein dihydropteroate synthetase, an enzyme that catalyzes the formation of a precursor of folate derivatives called 6-hydroxymethyl–7,8-dihydroprotien. Next, *galT* codes for a protein involved in galactose metabolism as a subset of carbohydrate metabolism. Finally, *galK* codes for a protein that catalyzes the transfer of a phosphate group from ATP to D-galactose also as a part of galactose metabolism. Finding bacterial samples of *P. anaerobius* and *P. stomatis* proved to be challenging to find as well. In fact, the sample of *P. stomatis* was obtained from Europe. Much of the recipe was designed from Dr. McKillip's suggestion and experience with past projects regarding PCR.

At this point we were ready to begin the meat of our research. The timeframe of our research coincided with the beginning of the spring semester 2018, a semester in which Spencer would spend studying abroad in Argentina. While we remained in contact throughout the semester, most of the research fell to me during the semester. We had decided to test each of the three primer sequences in triplicate, meaning that PCR was run on each primer in a series of three trials. Prior to the first trial being run, and whenever it was necessary, DNA samples had to be extracted from our sample bacteria and measured for quality. It is usually a good idea to perform the process several times to prove the reproducibility of your results throughout the

experiment. It just gives your data another level of appropriateness when your methods are scrutinized.

Research had its immediate challenges. This is not altogether surprising as many times the components of PCR need to be adjusted to give good and reproduceable results. A large issue at the beginning of the research-gathering process was that the melting temperatures were not as clear as they could be, and the graphs ended up being a bit jumbled. This led into the first of several trials that had to be re-done. At the suggestion of Dr. McKillip we tried a few steps to optimize the PCR recipe, specifically upping the amount of magnesium chloride we used in the solution in order to enhance the DNA polymerase activity and increase the robustness of the overall reaction. This led to improved results, but this change would not be the last we made throughout the research process.

One strange result that began cropping up in March that needed to be rectified was that the "Negative" tube was showing some fluorescence in our results. This was not supposed to be the case because the Negative tube should have no DNA sample for our SYBR green to attach to and fluoresce. Somehow, during my micro pipetting of these trials, some sample of *P. anaerobius*, *P. stomatis*, or really anything was contaminating this tube so I needed to adjust my procedure. It was a simple fix, really, that focused more on being patient and taking more time with each trial. For each subsequent trial, I readied the Negative tube first before any of my experimental tubes or the positive control tube. This, and the sterile environment under the hood I was working in, would guarantee that no contaminate could wind up in the Negative tube.

Another problem arose with the quality of the DNA samples we collected from our bacteria samples. They were of decent quality but an improvement in this aspect could lead to improved results in our trials. The fix to this problem was a fairly simple one, just an addendum

to the DNA extraction process I had followed before. The idea was to pass the sample through a specialized tube toward the end of the extraction that is specially designed to attract DNA material. This helps prevent any loss of material in the process of extraction, so we have more to work with when we are running our trials. It was after this adjustment that we started seeing much improved results in our trials as compared to our results we had toward the beginning of gathering research.

Overall, I was really happy with our research process. The different adjustments made to the DNA extraction and the PCR recipe led to better results, which is always a relief to see. I appreciated the flexibility of our process and our timeframe which allowed us to repeat trials as needed to obtain better data. The need, however, to perform several trials over again was consistently nerve-racking. What people not familiar with the PCR process might be interested to learn is how methodical the procedure really is.

To put it in perspective, to make everything in the "recipe" one needs to be micro pipetting, many times only a single microliter of "ingredient," into several small centrifuge tubes. So, there are many things that go into the recipe and constitute such a small amount of what will go into the tube, many times what seems to only be a drop of liquid – and there are upwards of 4-8 tubes that need to be made for the trial. All at the same time, the researcher needs to be vigilant and careful of what he or she is adding to each tube, what he or she has already added to the tube, and what he or she needs to add to the tube in the future. With varying amounts of materials and different materials altogether going into different tubes this can become a lot of information to keep track of at the same time and one mistake will ruin your results. I honestly can't remember how many times I had to scrap a tube because my thoughts wandered for a

moment or someone came into the lab to speak to me. It was always better to start over because if one tube had been incorrect during the trial, then I would be redoing the entire trial later.

The above also does not even go into the range of emotions one feels when they actually perform the reaction. Firstly, you feel a sense of relief because you have everything you need ready, you have pipetted everything properly, and you are hopeful of good results. Now you might be hopeful, but even if you think your methods are appropriate and you have carried them out well there is still that fear and nervousness of your results not turning out the way you expect. It took just over three hours for my reaction to run its course in each trial so I had that time to sit on my hands and worry, which was never a very nice time for me. There is a bit of a tradeoff later, though, when you come back to the computer and see that you have done everything correctly and you are getting good data, which is definitely a huge relief.

At the beginning of this project I would have never expected it to take so long and here now, at the end of it all, I feel only appreciation for the people who helped get me to this point. The senior Honor's thesis was something I had worried about ever since I had entered the college and I was extremely fortunate for such an excellent opportunity to fall into my lap. Dr. McKillip has been amazing throughout this process as he was always helpful and willing to supply suggestions to improve our results during the experimental phase. Spencer has been fantastic as well. He is a dependable person by nature and was always committed to what was, to him, an outside research project going on while he studied abroad. I feel nothing but appreciation for the pair of them and happiness for the opportunity to work with them and construct this project.

Unknown Bacteria: Differentiating between *Peptostreptococcus anaerobius* and *Peptostreptococcus stomatis* for the Clinic

Goodman, Mark S. and Stewart, Michael B.

Abstract

The cornerstone of this research was to differentiate between the closely-related species of *Peptostreptococcus anaerobius* and *Peptostreptococcus stomatis* from a nucleotide sequence perspective. This topic was decided because they have similar chemical properties that do not readily distinguish them (Holt, Sneath & Krieg, 1994) and they are difficult to culture in a timely manner (Murdoch, 1998). In the past, the solution to cases involving the genus *Peptostreptococcus* was to simply treat with one uniform amount of penicillin since the drug resolved cases involving both of these species of *Peptostreptococcus* (Minces et al., 2010). The problem with this action is the minimum inhibitory concentration (MIC) of antibiotic needed to kill *P. anaerobius* and *P. stomatis* differs for not just penicillin, but also a host of other drugs including metronidazole, clindamycin, and cefoxitin (Könönen et al., 2007). Due to these differences, the methods used in this experiment to differentiate *P. anaerobius* and *P. stomatis* included using the PCR technique to elongate three specific gene sequences found in *P. anaerobius* that are not found in *P. stomatis*. These gene sequences include *galT, folP,* and *galK*. The use of commercial PCR techniques such as the one performed will continue to increase, making the performed methods a valuable asset to the differentiation between these two species. Results of this PCR screening revealed that the melting temperatures for the designed primer sequences were not significantly different between the two species. These results indicate that the three chosen gene sequences are not appropriate for differentiation by melting temperature and, thus, new sequences need to be chosen and tested to further this line of research between these two species.

Introduction

Differentiation between *Peptostreptococcus anaerobius* and *Peptostreptococcus stomatis* is of great importance in the clinical realm. Specifically, there have been various links between gastrointestinal procedures and the occurrence of *Peptostreptococcus* induced endocarditis (Pathak et. al, 2013; Minces et al., 2010). There are more and diverse outcomes of these two species causing infection, explicated in more detail in the literature portion. The minimum inhibitory concentration for 50% inhibition of the species in question (MIC50) for *P. anaerobius* is significantly higher than the MIC50 for *P. stomatis* (Könönen et al, 2007). Distinguishing between these bacteria means patients potentially may be able to take in reduced amount of the antibiotic, thereby reducing the likelihood of adverse effects due to exposing the body to fewer exogenous products (Elding, 2012). A more defined need for the product leads to a more efficient clinic where antibiotics are not used in excess amounts. Based on background literature, such as Bergey's Clinical Bacteriology, the two species of *Peptostreptococcus* are not very well differentiated. The goal of this research was to differentiate between *P. anaerobius* and *P. stomatis* in order to establish information in the literature as a means to differentiate them. It was predicted that using these designed primer sequences to measure their melting temperatures would reveal that *P. anaerobius* would have an annealing temperature for the primer sequences due to its inclusion of the *galT*, *folP*, and *galK* genes, whereas there should not be an analogous annealing temperature for *P. stomatis* at these points. Furthermore, the intention of this research was to be used as a confirmatory test between whether the implicated bacteria is either of the species stomatis or

anaerobius. Future research may include discrete application in the realm of genitourinary-generated *Peptostreptococcus* spp. endocarditis.

Literature

The presence of the species *Peptostreptococcus stomatis* and *anaerobius* proliferates in many clinically-relevant areas in patients reporting to a medical clinic. Specifically, *P. stomatis* is implicated in the following pathologies: oral cavity infections, dentoalveolar abscesses and endodontic infections (Elizabeth Carmel Murphy & Inga-Maria Frick, 2012). *Peptostreptococcus anaerobius* is instead found in abdominal cavity infections, female urogenital tract infections and rarely infective endocarditis (Elizabeth Carmel Murphy & Inga-Maria Frick, 2012). In the case of infective endocarditis, it is known to infect prosthetic valves (Pathal et al., 2013)). This has implications in the necessity of further procedures to correct any prosthetic valve damage. As a further statement of the complexity of *Peptostreptococcus* in terms of being implicated in endocarditis, it has been reported that genitourinary infections predispose individuals to suffering from endocarditis due to this bacterium (Minces et al., 2010). As the literature shows, there is a need to recognize when this genus is the source of an infection in one of the various sites in the body. Furthermore, research shows that the differentiation in terms of the species is also clinically relevant in terms of the minimum inhibitory concentration (MIC) as will be discussed.

Numerous sources suggest that the MIC50 of *P. anaerobius* is higher than that of *P. stomatis* for several different antimicrobial agents. Before the two species were differentiated there were questions raised about the MIC values of microbes with *Peptostreptococcus* origins as, due to the lack of differentiation, it was unknown whether the penicillin used to treat the infection was the smallest effective dosage (Pathak et al., 2013). After the two species were found to be distinct, this led to a better understanding of why questions regarding MIC values formed and opened an opportunity for further research into determining these values for each *P. anaerobius* and *P. stomatis*. The following research delves into this question regarding MIC50 values for the two species for several antimicrobial agents (Byrk et al., 2007).

The data gathered in this research process fits well into the literature listed above as it furthers the knowledge compiled in the source. The literature listed above first discusses the areas of extraction of these species, eventually proceeding into the similarities between *P. anaerobius* and *P. stomatis* at length. Since the research focuses on the differences between the two at a superficial level in regards to their species, the research performed differentiated them on a more specific level in regards to their genetic composition. The literature explains the difficulties of differentiating between these species so research designed to perform this task will supplement knowledge already compiled on the two species. In future application other researchers may even use this new primer design to differentiate *P. anaerobius* and *P. stomatis* rapidly to further their own experiments. As discussed prior, their research would be made more difficult if there was no rapid way to differentiate these two species.

Gram-positive anaerobic cocci-commensals and opportunistic pathogens

Elizabeth Carmel Murphy & Inga-Maria Frick. Division of Infection Medicine, Department of Clinical Sciences, Lund University, Lund, Sweden. 2012 Federation of European Microbiological Societies. Published by John Wiley & Sons Ltd.

***Peptostreptococcus* Induced Native-Valve Endocarditis**

R Pathak, A Ngwe, H Enuh, J Saverimuttu. *Peptostreptococcus* Induced Native-Valve Endocarditis . The Internet Journal of Infectious Diseases. 2013 Volume 12 Number 1

***Peptostreptococcus* infective endocarditis and bacteremia. Analysis of cases at a tertiary medical center and review of the literature**

Minces LR, Shields RK, Sheridan K, Ho KS, Silveira FP. Anaerobe. 2010 Aug;16(4):327-30. doi: 10.1016/j.anaerobe.2010.03.011. Epub 2010 Apr 3.

Antimicrobial Susceptibilities of *Peptostreptococcus anaerobius* and the Newly Described *Peptostreptococcus stomatis* Isolated from Various Human Sources

Antimicrobial Agents and Chemotherapy, June 2007, p. 2205-2207. Volume 51, Number 6. American Society for Microbiology. Eija Könönen, Anne Bryk, Paivi Niemi, and Arja Kanervo-Nordstrom

Methods

DNA Extraction Procedure:

1. Grow bacterial culture at 32˚C for 12h shaking (density ~ 10^6 CFU/mL).
2. Pellet 1 mL of cells in a microcentrifuge at 10,000 rpm for 3 min. (RT). Discard supernatant into waste vessels at benches.
3. Resuspend cells in 200ml sterile TE by repeated pipetting; Add 30ml 10% SDS or 2.5ml proteinase K + 5ml lysozyme. Incubate 30-45 min. @ 37˚C (water bath in CL28). Periodically during the incubation, vortex momentarily to enhance cell wall disruption.
4. Boil 3-5 min. (or place tubes in a 90˚C water bath 5 min.); vortex 30sec.
5. Spin tube(s) for 2 min. @ 2K rpm, RT, to pellet cellular debris. Decant or pipet supernatant into a clean, labeled tube.
6. Add an equal volume of phenol (or chloroform) under hood; invert several times to mix very well. Centrifuge 10 min. @ 12K rpm (RT).
7. Carefully pipet top phase into a new labeled tube and add 0.1 vol. of cold 3M sodium acetate and 1 vol. of cold ethanol (95%). Mix by inversion.
8. Optional step: incubate tubes O/N at -20˚C
9. Centrifuge @ max. speed in microcentrifuge for 20-30 min. (use microfuge in CL40 and/or CL34. Temp. can be either RT or 4˚C.
10. Decant liquid phase into waste vessel and dry DNA pellet for spectrophotometric determination.

Components of PCR Trials:

Negative Tube:

25 mM $MgCl_2$	4 microliters
2x buffer	12.5 microliters
SYBR Green	1 microliter
Forward primer (exp.)	1 microliter
Reverse primer (exp.)	1 microliter
M-MvLv enzyme mix	1 microliter
Sterile Water	4.5 microliter
Total	25 microliters

Experimental Tube(s):

25 mM $MgCl_2$	4 microliters
2x buffer	12.5 microliters
SYBR Green	1 microliter
Forward primer (exp.)	1 microliter
Reverse primer (exp.)	1 microliter
M-MvLv enzyme mix	1 microliter
Sterile Water	(as needed)
DNA sample	(0.5 micrograms)
Total	25 microliters

Reference Tube:

25 mM $MgCl_2$	4 microliters
2x buffer	12.5 microliters
SYBR Green	1 microliter
Forward primer (exp.)	1 microliter
Reverse primer (exp.)	1 microliter
M-MvLv enzyme mix	1 microliter

Sterile Water	(as needed)
DNA sample	(0.5 micrograms)
Total	25 microliters

PCR primers used during the procedure appear as follows:

galK forward:

ATCAGGAAACACCAACTCCG

galK reverse:

TACCGCACCCCACCGCTGGG

folP forward:

accccagattcattttcaga

folP reverse:

atggcatagccagaaagag

galT forward:

tcaagtgacgggacatccga

galT reverse:

ttcgactagcatctatatcc

Results

	Trial 1 (°C)	Trial 2 (°C)	Trial 3 (°C)
galT Reference (PA) Trial_1:1	79.0	78.2	79.8
galT P. anaerobius Trial_1:2	77.4	75.2	78.2
galT P. stomatis Trial_1:3	78.8	78.5	76.1
folP Reference Trial_2:1	(PA) 78.8	(PS) 78.8	(PS) 74.5
folP P. anaerobius Trial_2:2	75.8	74.2	74.5
folP P. stomatis Trial_2:3	80.8	78.1	79.2
galK Reference (PA) Trial_3:1	75.8	80.8	77.5
galK P. anaerobius Trial_3:2	76.8	79.1	81.1
galK P. stomatis Trial_3:3	75.8	80.1	81.5

Table 1: Melting temperatures for each primer set in each trial performed. Reference sets used *P. anaerobius* and *P. stomatis* as available and are specified as *P. anaerobius* (PA) and *P. stomatis* (PS).

Note: In *folP* Reference Trial_2:1, the parenthetical initials indicate the reference species for each of the three experimental trials.

		N	Mean	Std. Deviation
Trial_1	1	3	78.03	1.966
	2	3	78.00	2.553
	3	3	76.67	.808
	Total	9	77.57	1.793
Trial_2	1	3	79.3667	1.25033
	2	3	78.9000	1.05830
	3	3	76.1667	2.58908
	Total	9	78.1444	2.14191
Trial_3	1	3	77.2667	2.65769
	2	3	78.9333	2.70986
	3	3	78.1667	3.65011
	Total	9	78.1222	2.73028

Table 2: Statistical difference as calculated by SPSS 24 one way ANOVA analysis within groups ($p < 0.05$). Each trial is separated, and primers are denoted as follows: Reference (1), *P. anaerobius* (2), and *P. stomatis* (3).

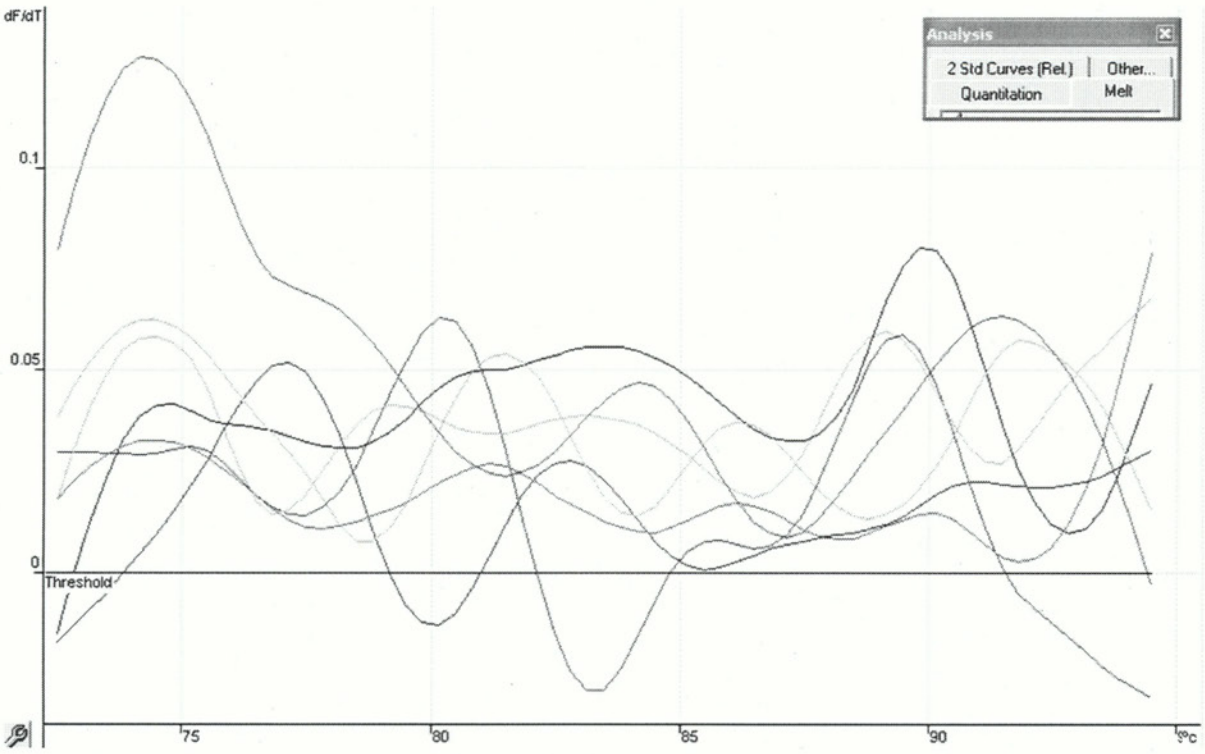

Figure 1: Raw melting temperature data from Trials 2 and 3 using the *galK* primer. Peaks found between 75-80 °C represent the point where the primers anneal to the specific gene sequence.

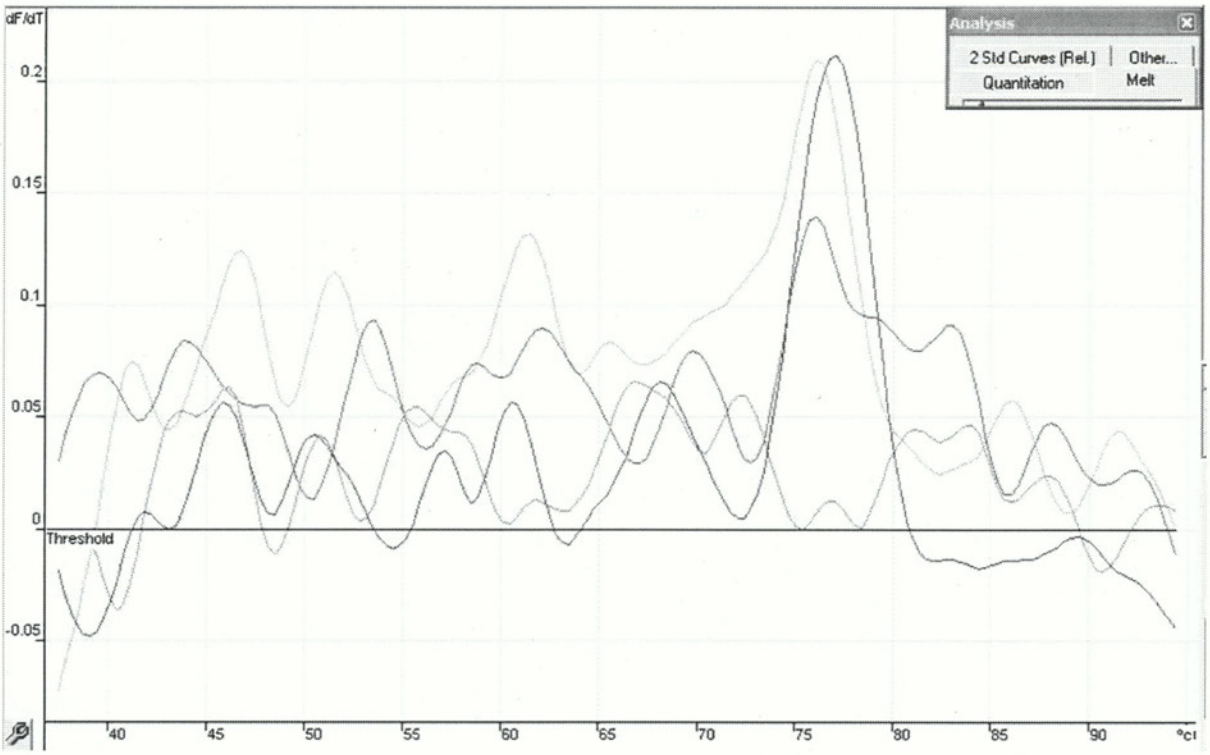

Figure 2: Raw melting temperature data from Trial 1 using the *galT* primer. Peaks found between 75-80 ℃ represent the point where the primers anneal to the specific gene sequence.

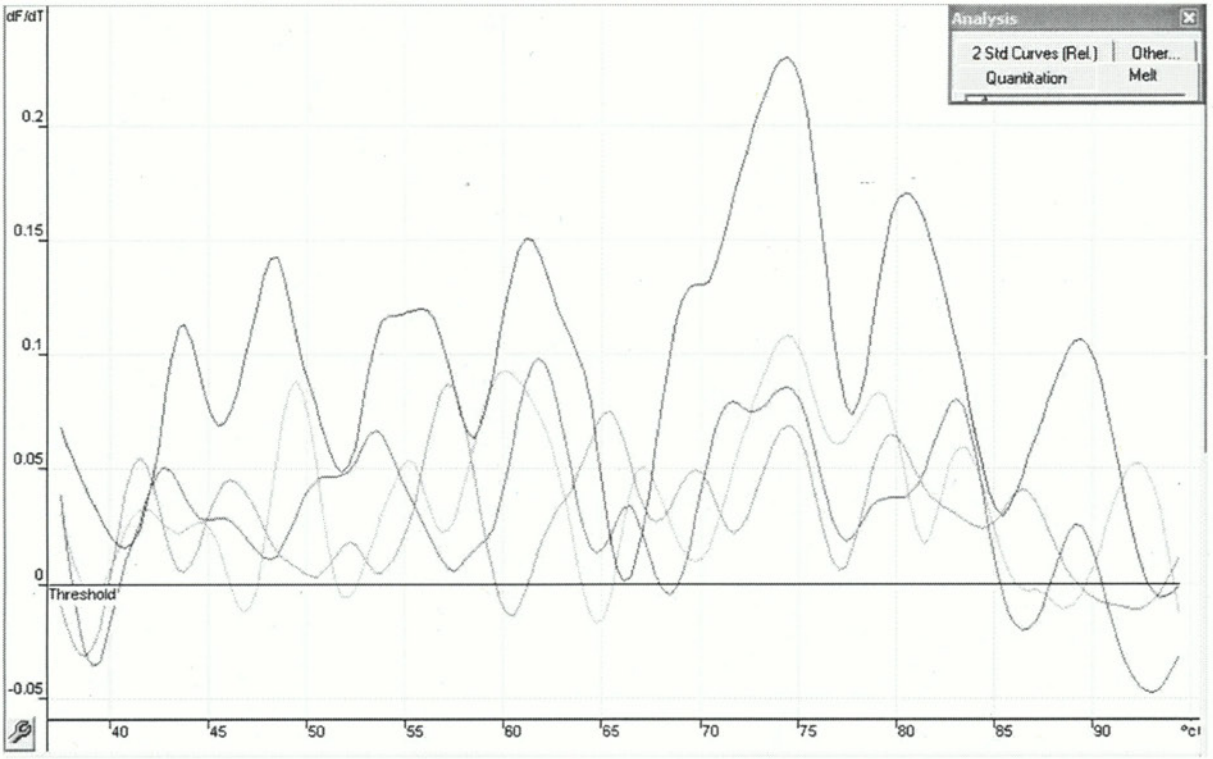

Figure 3: Raw melting temperature data from Trial 3 using the *folP* primer. Peaks found between 75-80 ℃ represent the point where the primers anneal to the specific gene sequence.

Discussion

The impact of differentiating the two bacteria *P. anaerobius* and *P. stomatis* lies in the fact each of these species of bacteria is implicated in various diseases for many different demographics. For example, according to one case study, *P. anaerobius* is implicated in sternal osteomyelitis, an infection that can stem from open wounds or surgical incisions (Y.-L. Chen et al, 2012). For example, *P. anaerobius* is implicated in bacterial vaginosis in women as well as endocarditis for men and women age 65 and older. In addition, *Peptostreptococcus stomatis* is implicated in periodontal diseases, gingivitis, and root canal infections (Murphy and Frick, 2012).

The MIC_{50} and MIC_{90} to kill *P. anaerobius* is several orders of magnitude greater than those for *P. stomatis* for the antibiotics amoxicillin, clindamycin, and metronidazole (Könönen, 2007). This is of economic and medicinal importance, then, to differentiate between the two species to understand which of these species is implicated in the disease in question. Specifically, in regards to the economic impact, the price of drug spending (which included antibiotics) increased over 200 dollars per hospital admission from 2013 to 2015 (Pollack and Kahn, 2016). Being able to differentiate these bacteria with greater ease and efficiency will potentially allow for a reduced but equally effective dose of an antibiotic.

In the clinical realm, the fewer outside substances exposed to the body is more desirable to prevent adverse side effects and this research is another key to making sure patients are treated with no more medication than is necessary to return them to their standard state of health (Weintraub,

2013).This basic science research has the potential to be quickly applied to medical facilities such as emergency departments for its practical use in caring for patients.

The results indicate that the gene sequences analyzed between the two species of *Peptostreptococcus* are not statistically different in terms of their melting temperatures. Such results are indicative that, in a limited view, these genes do not differentiate the species, and as such render our hypothesis invalid. In a broader scope this may lead to the conclusion that these species may not in fact be separate species but rather (whatever that word is to indicate they are different but not different enough to be distinct species). However, the previous research discussed in relation to their MIC values would contradict such conclusion. A more appropriate interpretation of this outcome can be analyzed in terms of the genes selected.

Concerning the genes selected, statistically the melting temperatures for the genes analyzed, *folP*, *galT*, and *galK*, were no different between *Peptostreptococcus anaerobius* and *Peptostreptococcus stomatis*. Such results present a difficult interpretation, as the literature indicated that these genes were physically not present within *P. stomatis*. Therefore, around the peaks of 75 to 80 degrees where these genes were recorded to occur in *P. anaerobius*, the melting temperatures for the analyzed sequence in *P. stomatis* should have been statistically different. Reasons to explain the disparity can include other gene sequences that would have the same melting temperatures as the genes being analyzed. Additionally, the primers may not have annealed to the correct gene sequences, thereby elongating genes that were not a part of the original hypothesis with similar melting temperatures.

Further research into this area of study could involve looking at other genes that have been reported to be present in *P. anaerobius* and not *P. stomatis*. An example of this type of gene would be fumB. Such an extension on the research reported in this paper would be significant due to identifying other genes that are more readily distinguished between these two species of bacteria taking into account the similar characteristics of surrounding genes to the target genes.

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